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Macrophages infected with amastigotes of *Leishmania major* and treated with IFN $\gamma$  *in vitro* develop potent antimicrobial activities that eliminate the intracellular parasite. This antileishmanial activity was suppressed in a dose dependent fashion by N<sup>G</sup>-monomethyl-L-arginine (N<sup>G</sup>MMLA), a competitive inhibitor of nitrite, nitrate, nitric oxide and L-citrulline synthesis from L-arginine. Excess L-arginine added to infected macrophage cultures reversed the inhibitory effects of N<sup>G</sup>MMLA. Addition of arginase to culture media inhibited intracellular killing by IFN $\gamma$ -treated cells. Similar effects were seen with macrophages obtained from BCG-infected C3H/HeN mice. Increased levels of nitrite, an oxidative product of the L-arginine dependent effector mechanism, was measured in cultures of infected IFN $\gamma$ -treated macrophages as well as infected BCG-activated macrophages. Nitrite production correlated with development of antileishmanial activity. Nitrite production and microbicidal activity both decreased when *in vivo* or *in vitro*-activated macrophages were cultured in the presence of either arginase or N<sup>G</sup>MMLA. Nitric oxide synthesized from a terminal guanidino nitrogen atom of L-arginine and a precursor of the nitrite measured, may disrupt Fe-dependent enzymatic pathways vital to the survival of amastigotes within macrophages.

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## ACTIVATED MACROPHAGES DESTROY INTRACELLULAR *Leishmania major* AMASTIGOTES BY AN L-ARGININE-DEPENDENT KILLING MECHANISM<sup>1</sup>

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Macrophages infected with amastigotes of *Leishmania major* and treated with IFN- $\gamma$  in vitro develop potent antimicrobial activities that eliminate the intracellular parasite. This antileishmanial activity was suppressed in a dose dependent fashion by N<sup>G</sup>-monomethyl-L-arginine (N<sup>G</sup>MMLA), a competitive inhibitor of nitrite, nitrate, nitric oxide and L-citrulline synthesis from L-arginine. Excess L-arginine added to infected macrophage cultures reversed the inhibitory effects of N<sup>G</sup>MMLA. Addition of arginase to culture media inhibited intracellular killing by IFN- $\gamma$ -treated cells. Similar effects were seen with macrophages obtained from BCG-infected C3H/HeN mice. Increased levels of nitrite, an oxidative product of the L-arginine-dependent effector mechanism, was measured in cultures of infected IFN- $\gamma$ -treated macrophages as well as infected BCG-activated macrophages. Nitrite production correlated with development of antileishmanial activity. Nitrite production and microbicidal activity both decreased when in vivo or in vitro-activated macrophages were cultured in the presence of either arginase or N<sup>G</sup>MMLA. Nitric oxide synthesized from a terminal guanidino nitrogen atom of L-arginine and a precursor of the nitrite measured, may disrupt Fe-dependent enzymatic pathways vital to the survival of amastigotes within macrophages.

Inasmuch as the *Leishmania* replicate only within macrophages of an infected host, elimination of the parasite and resolution of disease must invoke extraordinary metabolic changes in the infected macrophage. These changes shift the intracellular environment of the parasite from one that is supportive of replication, to one that is hostile to survival. Both in vitro and in vivo studies convincingly demonstrate that intracellular persistence of *Leishmania major* depends on the differentiative state of the macrophage at the onset of infection (1, 2) and the capacity of the macrophage to respond to T cell-derived

lymphokines during the course of disease (3-5). For example, macrophages from mice infected with the macrophage activating agent *Mycobacterium bovis* strain BCG are resistant to infection with amastigotes of *L. major*. Moreover, any parasites that do enter the BCG-activated macrophages are killed within 72 h (6). Immunotherapy with *M. bovis* may reflect a similar phenomenon in vivo: BCG admixed with *L. major* amastigotes and injected into susceptible mice blocks parasite replication in macrophages in tissues, and prevents development of both cutaneous lesions and lethal systemic disease (4, 7).

Resident peritoneal macrophages also develop antileishmanial activities in vitro after treatment with activating factors in lymphokines or recombinant IFN- $\gamma$ . With the appropriate in vitro activation signals, these cells develop resistance to infection with the parasite (8, 9), as well as the capacity to eradicate residual intracellular amastigotes of *L. major* (3). IFN- $\gamma$  clearly influences in vivo natural resistance to *L. major* infection in mice (10). Treatment of C3H/HeN mice with anti-IFN- $\gamma$  mAb at the time of inoculation of *L. major* alters the phenotype of resistant animals: they develop cutaneous lesions and systemic disease indistinguishable from that of genetically susceptible BALB/c mice. The exact role of IFN- $\gamma$  in clearance of amastigotes from the infected cutaneous tissues, however, has not been completely delineated.

The mechanism(s) by which activated macrophages kill intracellular amastigotes remain an enigma. The *Leishmania*, which replicate exclusively in phagolysosomes of macrophages, are under strong evolutionary pressure to develop compensatory mechanisms that subvert antimicrobial effector activities. The intracellular conversion of the sandfly-adapted promastigote to the amastigote form found in mammalian tissues is instrumental to the survival and propagation of the leishmanial species. Unlike the promastigote of *L. major*, the amastigote is quite resistant to the antimicrobial mechanisms of the unstimulated macrophage. For instance, attachment and entry of either the promastigote or the amastigote in macrophages elicits a superoxide synthesizing respiratory burst which the amastigote readily survives (2). The respiratory burst induced by the amastigote is of a lesser magnitude than that induced by the promastigote. Unlike the promastigote, *L. major* amastigotes are also less sensitive to cytokine-induced, oxygen-dependent antimicrobial mechanisms, such as H<sub>2</sub>O<sub>2</sub>. Although controversial, this relative resistance to reactive oxygen intermediates by intracellular tissue forms of the parasite may be due to their high levels of endogenous catalase, peroxidase, and superoxide dismutase.

Activated macrophages cause characteristic metabolic

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<sup>1</sup> In conducting the research in this report, the investigator adhered to the Guide for Laboratory Animal Facilities and Care as promulgated by the Committee of the Guide for Laboratory Animals and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council. The views of the authors do not purport to reflect the position of the department of the Army or the Department of Defense.

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changes in tumor target cells: inhibition of the citric acid cycle enzyme, aconitase (11, 12), and inhibition of mitochondrial respiration (12-15). Disruption of these vital metabolic pathways is independent of the respiratory burst (16, 17) and is mediated by L-arginine-derived inorganic nitrogen oxides produced by cytotoxic activated macrophages; in this case, nitric oxide appears to be the ultimate effector molecule (15, 18-20).

In this study, we report that synthesis of L-arginine-derived nitrite, an oxidative degradation product of nitric oxide, correlated with intracellular killing of *L. major* by activated macrophages. In addition, the competitive inhibitor of inorganic nitrogen oxide synthesis, N<sup>G</sup>-monomethyl-L-arginine (12, 15, 18, 19), blocked development of intracellular killing by macrophages activated with IFN- $\gamma$  in vitro and BCG in vivo. Intracellular killing of *L. major* by activated macrophages may be mediated by the same L-arginine dependent effector mechanism observed to cause cytostasis, and under certain conditions cytotoxicity, of mammalian neoplastic cells.

#### MATERIALS AND METHODS

**Animals.** Specific pathogen-free male C3H/HeNHS mice were obtained from Harlan Sprague-Dawley, Indianapolis, IN; BALB/cByJ mice were purchased from The Jackson Laboratory, Bar Harbor, MA. All animals were housed in barrier facilities until use, and were routinely screened and found to be negative for bacterial pathogens and for subclinical viral infections through the serodiagnostic services of Microbiological Associates, Bethesda, MD.

**Target organism.** The target microorganism used in these studies was the amastigote (tissue form) of the protozoan parasite, *L. major* (NIH strain 173). Parasites were maintained by passage in footpads of BALB/cByJ mice. Monodispersed amastigotes were obtained by disruption of infected footpad tissue and passage through no. 50 stainless steel mesh screens into DMEM (formula no. 78-0176, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems, Logan, UT) and 50  $\mu$ g/ml gentamicin (MA Bioproducts, Rockville, MD). Amastigotes were released from infected macrophages by homogenization in a Ten Broek tissue homogenizer; tissue debris was removed by centrifugation of the suspension at 200  $\times$  g. Parasites were stained with fluorescein diacetate and ethidium bromide, and were counted in a hemacytometer (24). Amastigote preparations were adjusted to 5  $\times$  10<sup>6</sup> viable organisms/ml before use.

**Infection of mice with *M. bovis*, strain BCG.** C3H/HeN mice were inoculated i.p. with 10<sup>5</sup> CFU of the Montreal strain of *M. bovis* strain BCG. Cells were harvested from the peritoneal cavity after 8 to 10 days of infection.

**Macrophages.** Resident PC<sup>3</sup> from untreated C3H/HeN mice were collected after i.p. injection of 8 to 10 ml of DMEM with 2% heat inactivated FCS and 50  $\mu$ g/ml gentamicin. Peritoneal fluids were pooled and samples were removed for differential and total cell counts; the remaining fluids were centrifuged at 200  $\times$  g for 10 min at 4°C. Differential cell counts were made on Wright-stained cell smears (Diff-Quick, Dade Diagnostics, Aquado, PR) prepared by cytocentrifugation (Cytospin centrifuge, Shandon Southern Instruments, Camberly, England); resident PC preparations usually contained 50  $\pm$  3% macrophages, 45  $\pm$  3% lymphocytes, 3  $\pm$  2% polymorphonuclear leukocytes, and 2  $\pm$  2% mast cells. Washed PC suspensions were adjusted to 1  $\times$  10<sup>6</sup> macrophages/ml in DMEM with 2% FCS and 50  $\mu$ g/ml gentamicin.

**Lymphokines.** IFN- $\gamma$  was supplied by Genentech, South San Francisco, CA. The concentration of IFN- $\gamma$  (U/ml) was confirmed by ELISA with the anti-IFN $\gamma$  mAb H1, developed and generously supplied by Dr. Robert Schreiber, Washington University School of Medicine, St. Louis, MO (25).

**Treatment of macrophages for induction of microbicidal activities against *L. major* amastigotes.** Macrophages were exposed to one amastigote/macrophage (1  $\times$  10<sup>6</sup> macrophages/ml; 0.5 ml/tube) for 2 h at 37°C, 5% CO<sub>2</sub> with periodic shaking, and then treated with IFN- $\gamma$  in the presence or absence of N<sup>G</sup>MMLA, (Calbiochem-Behring Corp., La Jolla, CA), bovine liver arginase (Sigma Chemical Co., St. Louis, MO), or L-arginine (Sigma). After 72 h of incubation at 37°C, aliquots of the cell suspensions were cytocentrifuged (700

$\times$  g, 7 min) and the percent macrophages with intracellular amastigotes was estimated by microscopic examination of stained cell smears. Results were expressed as mean percent *Leishmania*-infected macrophages  $\pm$  SEM for four to eight observations on two to four cultures (800 to 1600 macrophages observed). Microbicidal activity was defined as percent decrease in infected macrophages in treated cultures compared to control cultures, by the following formula: (% infected control macrophages - % infected treated macrophages)  $\times$  100  $\div$  % infected control macrophages.

**Measurement of NO<sub>2</sub><sup>-</sup> production by *L. major*-infected macrophages.** Cell-free culture fluids were assayed for nitrite by the Griess reaction (26). Briefly, 50- $\mu$ l aliquots of the conditioned medium were incubated with 200  $\mu$ l of 1% sulfanilamide and 200  $\mu$ l of 0.1% N-1-naphthylethylenediamine dihydrochloride in 2.5% H<sub>3</sub>PO<sub>4</sub> (Sigma) at room temperature for 5 min. Absorbance at 543 nm was measured. NO<sub>2</sub><sup>-</sup> was quantified by comparison to Na(NO<sub>2</sub>) as standard.

#### RESULTS

**Effects of methylated-L-arginine derivative on IFN- $\gamma$ -induced macrophage antimicrobial activities.** By 72 h, macrophages cultured with IFN- $\gamma$  develop the capacity to restrict replication of *L. major* amastigotes; the majority of these cells (>75%) actually kill the intracellular parasite (5). We examined the effects of N<sup>G</sup>MMLA, a metabolic inhibitor of inorganic nitrogen oxide synthesis from L-arginine (12, 15, 18, 19) on IFN- $\gamma$ -induced antimicrobial activities (Figs. 1 and 2). IFN- $\gamma$  at  $\geq$  5 U/ml induced 75 to 80% microbicidal activity in resident PC (Fig. 1); this IFN- $\gamma$ -induced intracellular destruction of amastigotes, however, was inhibited in a dose-dependent fashion by treatment of the cells with N<sup>G</sup>MMLA. Half-maximal activity of the competitive L-arginine inhibitor calculated by the N<sup>G</sup>MMLA dose response on 5 U/ml IFN- $\gamma$ -treated cells was 0.025  $\mu$ M.

Microbicidal activity in Figure 1 was estimated by noting the percent decrease in the number of infected cells in IFN- $\gamma$ -treated cultures compared to medium-treated controls, regardless of the number of amastigotes/infected cell. Microbicidal activity calculated in this manner underestimates the effects of IFN- $\gamma$  by ignoring cytostatic activity induced in cells unable to completely

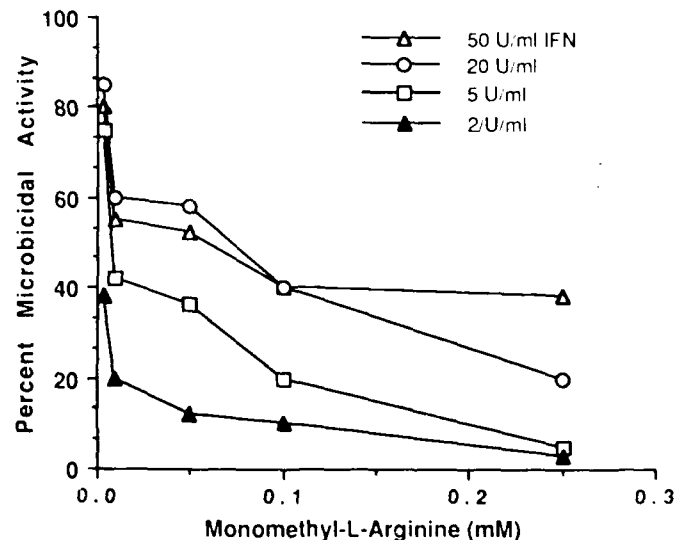


Figure 1. Effect of IFN- $\gamma$  (IFN) and monomethyl-L-arginine on macrophage antileishmanial activities. Resident macrophages (1  $\times$  10<sup>6</sup>/ml) were exposed to amastigotes (1  $\times$  10<sup>6</sup>/ml) for 2 h, and then treated with different concentrations of IFN- $\gamma$ , with or without monomethyl-L-arginine. After 72 h, 200- $\mu$ l aliquots were removed for cytocentrifugation and stained for light microscopy. Percent infected macrophages was determined by observation of 400 macrophages per treatment group. Percent microbicidal activity was calculated by determining the change in percentage of infected macrophages in treated cultures compared to control, medium-treated cells as described in Materials and Methods.

<sup>3</sup> Abbreviation used in this paper: PC, peritoneal cells; N<sup>G</sup>, monomethyl-L-arginine.

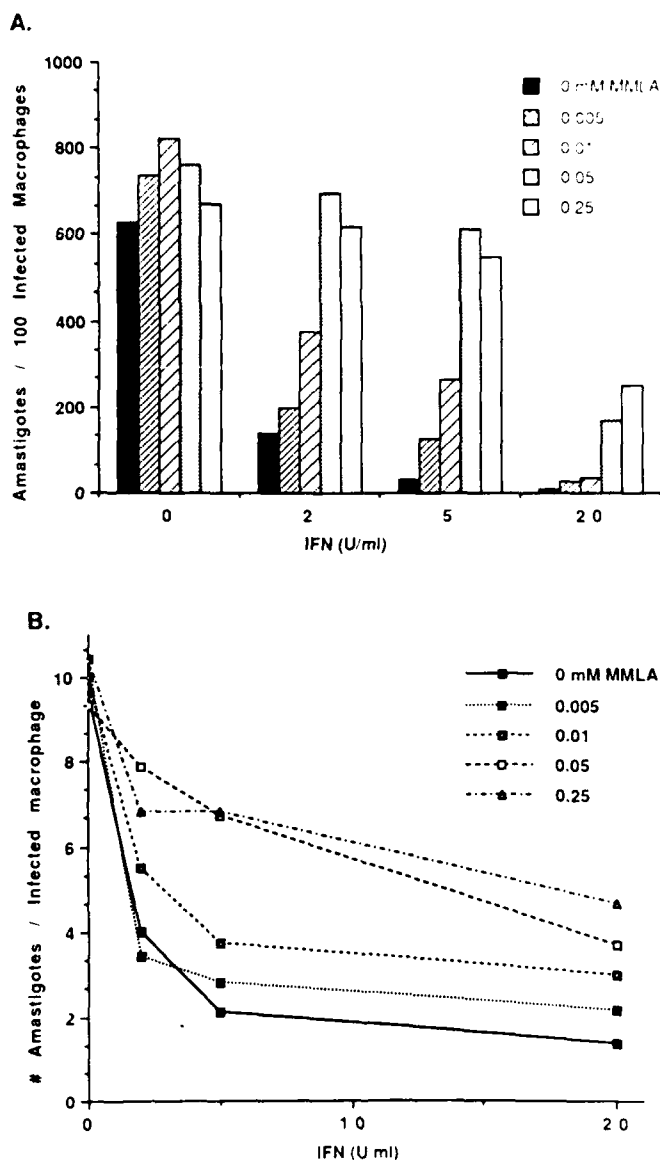


Figure 2. Effect of monomethyl-L-arginine (MMLA) on intracellular killing of amastigotes by IFN- $\gamma$ -activated macrophages. Resident macrophages ( $1 \times 10^6$ /ml) were exposed to amastigotes ( $1 \times 10^6$ /ml) for 2 h, and then treated with different concentrations of IFN- $\gamma$ , with or without MMLA. After 72 h, 200- $\mu$ l aliquots were removed for cytocentrifugation and stained for light microscopy. A, The number of intracellular amastigotes was determined by observation of 400 macrophages per treatment group. Results are expressed as amastigotes per 100 macrophages. Each value is the mean of two determinations on duplicate samples. B, Intracellular replication of amastigotes was estimated by calculating the mean number of amastigotes/infected macrophage.

clear the parasite. To examine whether N<sup>G</sup>MMLA influenced the ability of the parasite to replicate inside of cells, we determined the number of amastigotes per 100 macrophages after 72-h incubation in the presence or absence of IFN- $\gamma$  and N<sup>G</sup>MMLA (Fig. 2A). The number of parasites in N<sup>G</sup>MMLA-treated macrophages was greater than in untreated control cells. These results suggest that untreated macrophages exerted a baseline level of cytostasis which could be blocked by inhibiting intrinsic L-arginine-dependent metabolic pathways with the N<sup>G</sup>MMLA. The mean number of amastigotes per infected macrophage in control cultures with or without N<sup>G</sup>MMLA (Fig. 1B) ranged from 9.2 (untreated) to 10.5 (with N<sup>G</sup>MMLA). The number of amastigotes in all IFN- $\gamma$ -treated cultures exposed to  $\geq 0.01$  mM N<sup>G</sup>MMLA, calcu-

lated by either amastigotes/100 macrophages (Fig. 2A) or mean amastigotes/infected macrophage (Fig. 2B), were markedly different than the number of amastigotes in cells treated only with IFN- $\gamma$ . After 72 h, a mean of 10 amastigotes was observed in each infected control cell (Fig. 2B). In contrast, a mean of only two amastigotes were found in each IFN- $\gamma$ -treated residually infected macrophage. This number is not significantly different than the number of intracellular amastigotes in infected cells at the outset (time 0) of infection (1.6 amastigotes/infected cell). Thus, IFN- $\gamma$  treatment of macrophages resulted in cytostatic, as well as cytotoxic activity against the amastigote. When infected IFN- $\gamma$ -treated cells were cultured in as little as 0.01 mM N<sup>G</sup>MMLA, however, the number of amastigotes per infected cell nearly doubled, and in some cases, tripled (mean for all residually infected macrophages in IFN- $\gamma$ -treated cultures of 5.1 amastigotes/cell).

**Effects of N<sup>G</sup>MMLA on antimicrobial activity of macrophages activated in vivo.** Intraperitoneal infection of C3H/HeN mice with *M. bovis* strain BCG induces a population of macrophages activated to kill *L. major* amastigotes (6, 7). The addition of N<sup>G</sup>MMLA to cultures of these in vivo-activated macrophages inhibited antimicrobial activity by more than 95% (Fig. 3). This level of inhibition was similar to that observed with macrophages activated by IFN- $\gamma$  in vivo.

**Specificity of inhibitory effect of N<sup>G</sup>MMLA.** The inhibitory effect of N<sup>G</sup>MMLA on IFN- $\gamma$ -induced intracellular killing was reversed by increasing the concentration of L-arginine in the culture medium (Table I). The addition of 1 to 2 mM L-arginine totally reversed the inhibitory effect of 0.1 mM of the methylated derivative. Excess arginine alone had no effect (data not shown).

Macrophages were infected with amastigotes and exposed to IFN- $\gamma$  in DMEM that contained various concentrations of arginase (Fig. 4), an enzyme which converts L-arginine to L-ornithine and urea. Neither of these metabolic products of L-arginine are adequate substrates for nitric oxide production by activated macrophages (12, 18). Under these conditions, infected macrophages remained viable in culture in excess of 72 h. The antileishmanial activity normally expressed by IFN- $\gamma$ -treated macrophages was inhibited by exogenously added arginase (Fig. 4). These results suggest that depletion of extracellular pools of L-arginine, a necessary substrate for inorganic nitrogen oxide synthesis, blocks development of potent intracellular killing mechanisms by IFN- $\gamma$ -treated macrophages.

**Correlation of anti-leishmanial activity with L-arginine-derived NO<sub>2</sub><sup>-</sup> production by activated macrophages.** We examined the production of NO<sub>2</sub><sup>-</sup>, a degradation product of nitric oxide, the postulated effector molecule derived from L-arginine oxidation (18–21). NO<sub>2</sub><sup>-</sup> detection provides a stable and reliable marker for the L-arginine-dependent effector mechanism (15, 16, 18, 19). As shown in Table II, expression of intracellular killing by IFN- $\gamma$ -treated, *L. major*-infected macrophages correlated with NO<sub>2</sub><sup>-</sup> production as measured by the Greiss reaction (26). Further, both NO<sub>2</sub><sup>-</sup> production and microbicidal activity by IFN- $\gamma$ -treated, *L. major*-infected macrophages were blocked with either arginase or N<sup>G</sup>MMLA that has been shown to inhibit the cytotoxicity of activated macrophages for neoplastic cells (12, 18). Similar correlation of nitrite production and intracellular killing

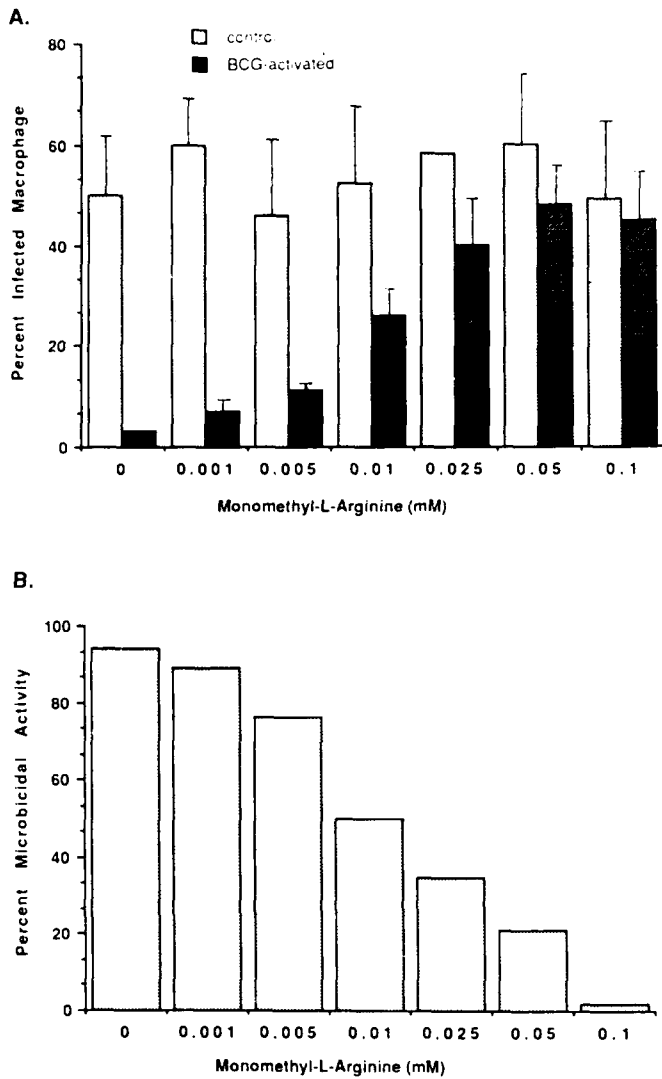


Figure 3. Effects of monomethyl-L-arginine on antileishmanial activity of macrophages obtained from BCG-infected mice. Resident macrophages and macrophages for BCG-infected mice ( $1 \times 10^6$ /ml) were exposed to amastigotes ( $1 \times 10^6$ /ml) for 2 h, and then treated with different concentrations monomethyl-L-arginine. After 72 h, 200- $\mu$ l aliquots were removed for cytocentrifugation and stained for light microscopy. A. Percent infected macrophages  $\pm$  SEM was determined by observation of 400 macrophages per treatment group. B. Percent microbicidal activity was calculated by determining the change in percentage of infected macrophages in treated cultures compared to control, medium-treated resident cells. Results are a representation of three experiments.

TABLE I  
Effects of N<sup>G</sup>MMLA on IFN- $\gamma$ -induced intracellular killing in presence of excess L-arginine<sup>a</sup>

Cells Incubated 72 H in	Infected macrophages (%) $\pm$ SEM	Microbicidal Activity (%)
Medium	53 $\pm$ 6	
+ 0.1 mM N <sup>G</sup> MMLA	58 $\pm$ 4	0
20 U/ml IFN- $\gamma$	3 $\pm$ 1	94
20 U/ml IFN- $\gamma$ + 0.1 mM N <sup>G</sup> MMLA	35 $\pm$ 8	34
+ 0.1 mM N <sup>G</sup> MMLA + 1.4 mM L-arginine	8 $\pm$ 3	84
+ 2.4 mM L-arginine	2 $\pm$ 2	96

<sup>a</sup> Exogenous L-arginine was added to culture media at the time that L. major-infected macrophages were treated with IFN- $\gamma$  and N<sup>G</sup>MMLA. Determination of the percentage of infected cells and microbicidal activity is described in Materials and Methods.

<sup>b</sup> Basic medium contains 0.4 mM L-arginine to which other supplements were added.

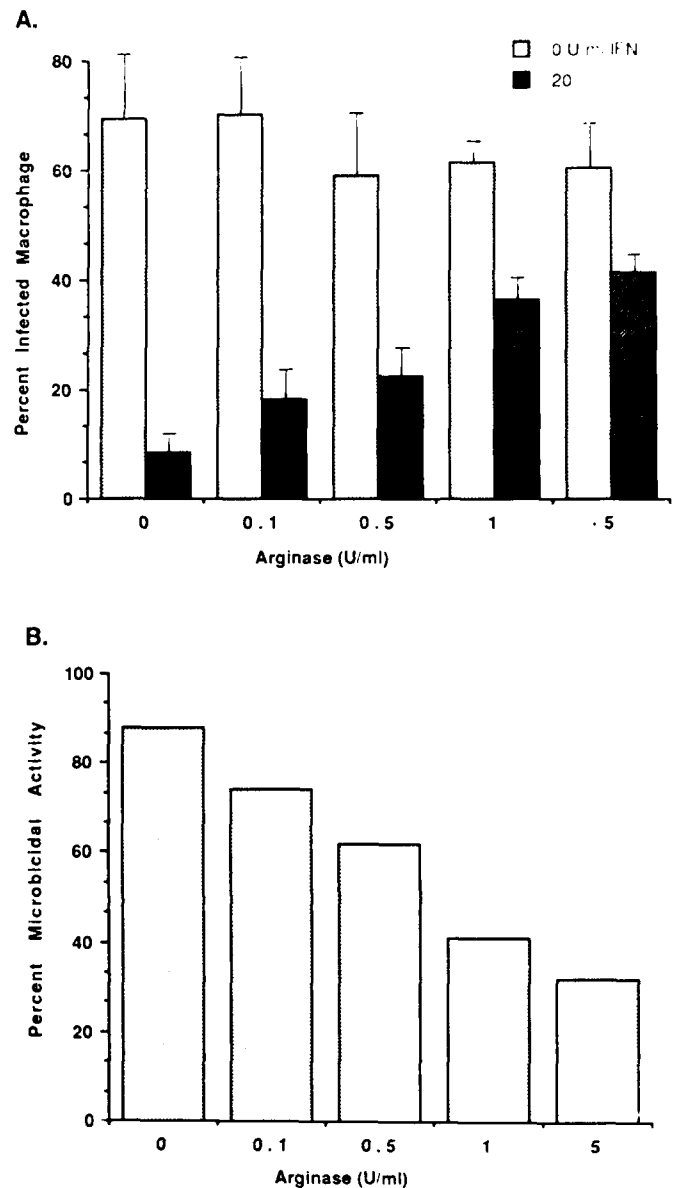


Figure 4. Effect of arginase on amastigote intracellular survival in IFN- $\gamma$ -treated macrophages. Resident macrophages ( $1 \times 10^6$ /ml) were exposed to amastigotes ( $1 \times 10^6$ /ml) for 2 h, and then treated with 20 U/ml IFN- $\gamma$  in the presence of various concentrations of bovine liver arginase. After 72 h, 200- $\mu$ l aliquots were removed for cytocentrifugation and stained for light microscopy. A. Percent infected macrophages  $\pm$  SEM was determined by observation of 400 macrophages per treatment group. B. Percent microbicidal activity was calculated by determining the change in percentage of infected macrophages in treated cultures compared to control, medium-treated cells as described in Materials and Methods.

TABLE II  
Correlation between NO<sub>2</sub><sup>-</sup> production and microbicidal activity by IFN- $\gamma$ -stimulated macrophages<sup>a</sup>

Cells Treated 72 H with	Microbicidal Activity (%)	NO <sub>2</sub> <sup>-</sup> ( $\mu$ M/10 <sup>6</sup> Cells 72 H)
0 U/ml IFN- $\gamma$		>1 $\pm$ 2
10 U/ml IFN- $\gamma$	90	65 $\pm$ 11
+ 1 mM N <sup>G</sup> MMLA	4	1 $\pm$ 0
+ 5 U/ml arginase	30	8 $\pm$ 5

<sup>a</sup> After 72 h, treated macrophage cultures were centrifuged, and cell pellets were resuspended in 0.5 ml fresh medium. Culture fluids were assayed for NO<sub>2</sub><sup>-</sup> by Greiss reaction (26). Cell smears were prepared by cytocentrifugation for assessment of microbicidal activity. Both assays are described in Materials and Methods.

occurred in macrophages for BCG-infected C3H/HeN mice treated in culture with various concentrations of N<sup>G</sup>MMLA (Table III).

TABLE III

$\text{NO}_2^-$  levels in culture fluids of *L. major*-infected macrophages from BCG-inoculated C3H/HeN mice treated with  $\text{N}^{\text{G}}$ MMLA<sup>a</sup>

$\text{N}^{\text{G}}$ MMLA (mM)	Infected Macrophages (% $\pm$ SEM)	Microbicidal Activity (%)	$\text{NO}_2^-$ ( $\mu\text{M}/10^6$ Cells/72 H)
0	6 $\pm$ 4	89	80
0.01	8 $\pm$ 3	85	67
0.05	36 $\pm$ 1	35	50
0.10	46 $\pm$ 6	16	42
0.25	51 $\pm$ 4	7	24
0.50	53 $\pm$ 4	4	16
Macrophages from uninoculated mice (normal control):			
0	55 $\pm$ 3		2

<sup>a</sup> After 72 h, treated macrophage cultures were centrifuged (500  $\times$  g, 10 min), and cell pellets were resuspended in 0.5 ml fresh medium. Culture fluids were assayed for  $\text{NO}_2^-$  by the Greiss reaction (26). Cell smears were prepared by cytocentrifugation for assessment of microbicidal activity.

## DISCUSSION

This report provides strong evidence for an L-arginine-dependent effector mechanism in the intracellular killing activities of macrophages activated by IFN- $\gamma$ : (1)  $\text{N}^{\text{G}}$ MMLA, a competitive inhibitor of the synthesis of inorganic nitrogen oxides derived from L-arginine (12, 15, 18, 19), inhibited intracellular killing of amastigotes by both IFN- $\gamma$ -treated and BCG-activated macrophages; (2) an excess of exogenous L-arginine reversed the inhibitory effects of  $\text{N}^{\text{G}}$ MMLA; (3) addition of arginase to the culture medium of amastigote-infected, IFN- $\gamma$ -treated macrophages suppressed antimicrobial activity; and (4)  $\text{NO}_2^-$  production correlated with intracellular destruction of amastigotes, and was inhibited by  $\text{N}^{\text{G}}$ MMLA and arginase. Collectively, these data suggest that nitric oxide synthesized from the terminal guanidino nitrogen atom of L-arginine plays a major role as the effector molecule in intracellular destruction of *L. major* amastigotes by activated macrophages.

The actual intracellular signals required for production of nitrite, nitrate, and nitric oxide by mammalian cells are incompletely understood, but these effector molecules are clearly regulated independently of the respiratory burst and reactive oxygen intermediates (16, 17, 28, 29). Our data on nitrite synthesis and release by activated macrophages (data not shown) suggest that secretion of  $\text{NO}_2^-$  at levels sufficient to affect viability of intracellular amastigotes after stimulation with IFN- $\gamma$  alone does not occur in uninfected macrophages. Yet cells activated in vitro in the studies reported above were treated with a single activation agent, IFN- $\gamma$ . How then does the IFN- $\gamma$ -activated macrophage receive a second signal for intracellular destruction of *L. major* amastigotes? It is known that certain microbial products (i.e., LPS and muramyl dipeptide) function as co-signal in the induction of inorganic nitrogen oxides synthesis for L-arginine by activated macrophages (30).

The precise nature of the metabolic changes in the amastigote induced by inorganic nitrogen oxides that result in death of the parasite are unknown. Several effects of L-arginine catabolism in other systems, however, may shed light on the intracellular fate of the amastigote. For example, Iyengar et al. (17) speculates that N-guanido-hydroxylated arginine, a postulated intermediate in the conversion of L-arginine to nitrite/nitrate and L-citrulline, is a potent inhibitor of DNA synthesis. Inasmuch as N-hydroxylated guanidines possess antitumor and microbicidal activity, it is possible that such

derivatives inhibit DNA synthesis, and are the proximal cause of amastigote cytolysis in IFN- $\gamma$ -treated macrophages.

The generation of nitric oxide by activated macrophages also induces the heme-dependent activation of guanylate cyclase, with the subsequent stimulation of the secondary messenger, cGMP. This may, in turn, serve as a signal for monokine production involved in the effector function, and the release of potent effector molecules yet to be identified. IFN- $\gamma$  induction of such a pathway in macrophages would parallel the acetylcholine-induced production of endothelium-derived relaxation factor, which closely resembles nitric oxide (31, 32). Endothelium-derived relaxation factor causes vascular smooth muscle relaxation and inhibition of platelet adhesion by activating guanylate cyclase, elevating intracellular cyclic GMP, and causing  $\text{Ca}^{2+}$  efflux. Similar events may take place in the activated macrophage: increased levels of cGMP, followed by changes in intracellular  $\text{Ca}^{2+}$ , could initiate the cascade of events involved in the macrophage effector activity.

A third possibility, and one which we presently favor, is that the short-lived precursor of nitrite synthesis, nitric oxide, may be the actual effector molecule that causes stasis and/or lysis of the intracellular parasite. Nitric oxide is synthesized from a terminal guanidino nitrogen atom of L-arginine by activated macrophages (19, 21), and could explain loss of intracellular iron loss (11, 15, 33) and inhibition of enzymes with iron-sulfur prosthetic groups that are important in a number of vital metabolic pathways of tumor targets (11, 12, 18–20) and in certain microorganisms. Cytotoxic activated macrophages inhibit two oxidoreductases of the mitochondrial electron transport chain in tumor targets (13, 14), the citric acid cycle enzyme aconitase (11, 12, 15, 18, 19), and DNA replication (11, 12, 18). Each of the enzymes have a catalytically active iron linked to a sulfur group that could be degraded by nitric oxide, and released as an iron-nitrosyl complex. Nitric oxide gas mimics macrophage-mediated tumor cytotoxicity as measured by [ $^{55}\text{Fe}$ ] release (19), inhibition of mitochondrial aconitase activity and respiration (19, 20), and [ $^3\text{H}$ ] TdR uptake (19). In an analogous manner, nitric oxide generated in the IFN- $\gamma$ -treated, *L. major*-infected macrophage may cause iron efflux, followed by Fe-dependent enzyme inhibition in the amastigote.

Although detailed biochemical pathways for carbohydrate metabolism are not well characterized in the amastigote, both the promastigote and the amastigote do contain a mitochondrion, enzymes of the TCA cycle, and rely on mitochondrial electron transport and ATP synthesis, especially during transformation of the promastigote to amastigote (for review, see Ref. 22). It is tempting to speculate that inhibition of mitochondrial respiration by the L-arginine-dependent effector mechanism characterized in macrophage-mediated tumor injury (11–15) and fungistasis (23) may also be the mechanism for IFN- $\gamma$ -induced destruction of intracellular parasites by activated macrophages. One target within the amastigote may indeed be mitochondrial respiration, because leishmanias contain an antimycin A sensitive mitochondrion (22). Another candidate target enzyme is citric acid cycle aconitase, active in a number of *Leishmania* sp. including *L. major*. Finally, the superoxide dismutase found in *L. major* may be the Achilles heel of intracellular amas-

tigotes in the activated macrophage. This superoxide dismutase is distinctively different from the mammalian enzyme form: it is an Fe-dependent enzyme (2). Thus, activity of the iron-containing superoxide dismutase may be abolished by the loss of intracellular  $\text{Fe}^{++}$  induced by L-arginine-derived nitric oxide, and the actual effector molecule may be a reactive oxygen intermediate for which the parasite no longer has a defense. This may be further exacerbated by IFN- $\gamma$ -induced down-regulation of the transferrin receptor (34, 35). The combined effects of iron loss and decreased uptake may explain IFN- $\gamma$ -induced killing. It was recently reported that doubling the amount of iron-saturated transferrin in the culture medium can reverse the capacity of IFN- $\gamma$ -activated monocytes to inhibit intracellular *Legionella pneumophila* multiplication. In a similar fashion, medium supplemented with ferrous ion restores aconitase activity in macrophage injured-tumor target cells (11, 15, 33). Studies are currently underway to determine if amastigote intracellular survival is iron dependent.

It will not be easy to pinpoint the actual events that lead to amastigote death, because the amastigote is an obligate intracellular parasite of macrophages, and must be studied inside its host cell. Despite these inherent difficulties, however, further understanding of the biochemical pathway, regulation, and physiologic role of L-arginine-derived inorganic nitrogen oxides by activated macrophages is essential. This information will provide insight into the use of cytokines as immunotherapeutic modalities, and facilitate design of drugs that enhance nonspecific protective host immunity against infectious agents.

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